Journal of Dental Research
2020, Vol. 99(1) 98–106

DOI: 10.1177/0022034519885088
journals.sagepub.com/home/jdr

# HDAC3 Regulates Gingival Fibroblast Inflammatory Responses in Periodontitis

**K.B. Lagosz[1], A. Bysiek[1], J.M. Macina[1], G.P. Bereta[1], M. Kantorowicz[2], W. Lipska[2], M. Sochalska[1], K. Gawron[1], T. Kaczmarzyk[2,3], M. Chomyszyn-Gajewska[2], G. Fossati[4], J. Potempa[1,5], and A.M. Grabiec[1]**

**Abstract**

Histone deacetylases (HDACs) are important regulators of gene expression that are aberrantly regulated in several inflammatory and infectious diseases. HDAC inhibitors (HDACi) suppress inflammatory activation of various cell types through epigenetic and non-epigenetic mechanisms, and ameliorate pathology in a mouse model of periodontitis. Activation of gingival fibroblasts (GFs) significantly contributes to the development of periodontitis and the anaerobic bacterium *Porphyromonas gingivalis* plays a key role in driving chronic inflammation. Here, we analyzed the role of HDACs in inflammatory responses of GFs. Pan-HDACi suberoylanilide hydroxamic acid (SAHA) and/or ITF2357 (givinostat) significantly reduced TNFα- and *P. gingivalis*–inducible expression and/or production of a cluster of inflammatory mediators in healthy donor GFs (*IL1B, CCL2, CCL5, CXCL10, COX2*, and *MMP3*) without affecting cell viability. Selective inhibition of HDAC3/6, but not specific HDAC1, HDAC6, or HDAC8 inhibition, reproduced the suppressive effects of pan-HDACi on the inflammatory gene expression profile induced by TNFα and *P. gingivalis*, suggesting a critical role for HDAC3 in GF inflammatory activation. Consistently, silencing of HDAC3 expression with siRNA largely recapitulated the effects of HDAC3/6i on mRNA levels of inflammatory mediators in *P. gingivalis*–infected GFs. In contrast, *P. gingivalis* internalization and intracellular survival in GFs remained unaffected by HDACi. Activation of mitogen-activated protein kinases and NFκB signaling was unaffected by global or HDAC3/6-selective HDACi, and new protein synthesis was not required for gene suppression by HDACi. Finally, pan-HDACi and HDAC3/6i suppressed *P. gingivalis*–induced expression of *IL1B, CCL2, CCL5, CXCL10, MMP1,* and *MMP3* in GFs from patients with periodontitis. Our results identify HDAC3 as an important regulator of inflammatory gene expression in GFs and suggest that therapeutic targeting of HDAC activity, in particular HDAC3, may be clinically beneficial in suppressing inflammation in periodontal disease.

**Keywords:** epigenetics, inflammation, host-pathogen interactions, innate immunity, gene expression, cell biology

## Introduction

Inflammatory responses are pivotally regulated at the cellular level by reversible acetylation of histones and non-histone proteins. The tightly controlled equilibrium between acetylation and deacetylation of N-terminal lysine residues on histones, catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, is a critical component of epigenetic regulation (Bannister and Kouzarides 2011). Dysregulation of this process contributes to pathology of many diseases caused by aberrant activation of the immune system: alterations in histone acetylation marks and HAT/HDAC balance have been reported in rheumatoid arthritis (RA), asthma, chronic obstructive pulmonary disease and systemic lupus erythematosus (Zhang and Zhang 2015). The importance of protein acetylation in inflammatory responses has also been highlighted by the discovery that pathogenic bacteria target HDAC-dependent regulatory mechanisms to evade the host immune response (Grabiec and Potempa 2018).

Mammalian cells express 18 members of the HDAC family that consists of classical zinc-dependent HDACs and nicotinamide adenine dinucleotide (NAD)–dependent sirtuins (Sirt-1-7). Zinc-dependent HDACs are further divided into class I (HDACs 1-3 and 8), class IIa (HDACs 4-5, 7 and 9), class IIb (HDACs 6 and 10) and class IV (HDAC 11) that differ in their expression pattern, subcellular localization and enzymatic activity profile (Yang and Seto 2008). Small-molecule HDAC inhibitors (HDACi) have shown strong immunomodulatory

[1]Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland
[2]Department of Periodontology and Clinical Oral Pathology, Faculty of Medicine, Jagiellonian University Medical College, Kraków, Poland
[3]Department of Oral Surgery, Faculty of Medicine, Jagiellonian University Medical College, Kraków, Poland
[4]Italfarmaco, Cinisello Balsamo, Milan, Italy
[5]Department of Oral Immunology and Infectious Diseases, University of Louisville School of Dentistry, Louisville, KY, USA

A supplemental appendix to this article is available online.

**Corresponding Author:**
A.M. Grabiec, Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, Kraków 30-387, Poland.
Email: aleksander.grabiec@uj.edu.pl

properties in multiple cell types in vitro and in animal models of inflammatory diseases (Leoni et al. 2002; Leoni et al. 2005) suggesting that HDACi may be used to ameliorate pathology in conditions associated with acute or chronic inflammation (Grabiec et al. 2011). Importantly, the pan-HDACi vorinostat (suberoylanilide hydroxamic acid, SAHA) and givinostat (ITF2357) demonstrated a good safety profile and initial clinical efficacy in patients with graft-versus-host disease and systemic-onset juvenile idiopathic arthritis, respectively (Vojinovic et al. 2011; Choi et al. 2014).

Unrestrained production of inflammatory mediators by resident gingival cells and infiltrating immune cells plays a central role in the pathogenesis of periodontitis (Yucel-Lindberg and Båge 2013). Chronic inflammation is driven by microbial imbalance (dysbiosis) which triggers a local immune response to oral pathogens, among which the anaerobic bacterium *Porphyromonas gingivalis* plays a crucial role due to its high virulence and effective strategies of immune evasion (Hajishengallis 2014). In periodontitis, gingival fibroblasts (GFs), which are the most abundant resident gingival cells, are constantly exposed to oral pathogens and their products. This, in turn, leads to excessive production of cytokines, chemokines and matrix-degrading enzymes which significantly contribute to periodontal ligament destruction and alveolar bone resorption (Benakanakere and Kinane 2012).

Although meticulous studies on epigenetic and non-epigenetic HDAC functions and therapeutic potential of HDACi in chronic inflammatory diseases have been conducted, the involvement of HDACs in periodontitis pathogenesis remains poorly defined. HDAC expression profiling revealed elevated levels of HDAC1 colocalizing with tumor necrosis factor-α (TNFα)–expressing cells in the gingival tissue from patients with chronic periodontitis compared to controls (Cantley et al. 2016). In vivo, alterations in histone acetylation have been implicated in the development of ligature-induced periodontitis (Martins et al. 2016) and HDACi protected mice from alveolar bone loss in *P. gingivalis*–induced periodontitis (Cantley et al. 2011). However, the role of HDACs in GF responses to oral pathogens remains unknown. In this study, we used a panel of global and selective HDACi in combination with a gene silencing approach to analyze the role of HDAC activity in inflammatory activation of GFs in periodontitis, and to delineate the contributions of individual HDAC family members to this process.

## Materials and Methods

### *Subjects, Cell Isolation, and Culture*

Gingival tissue specimens were collected from patients with chronic periodontitis ($n = 4$) and from healthy individuals ($n = 9$) undergoing orthodontic treatment at the Department of Periodontology and Clinical Oral Pathology, Faculty of Medicine, Jagiellonian University Medical College, Kraków, Poland. The study was approved by the Bioethical Committee of the Jagiellonian University (permit numbers 122.6120.337 .2016 and KBET/310/B/2012). All subjects gave written informed consent in accordance with the Declaration of Helsinki before entering the study. Clinical characteristics of healthy donors and patients with periodontitis are shown in Appendix Table 1. Primary GFs were derived from gingival tissue samples and cultured as described previously (Maksylewicz et al. 2019), and were used for experiments between passages 4 and 9.

### *Bacterial Culture, Cell Infection, and Treatment with HDAC Inhibitors*

Wild-type *P. gingivalis* strain ATCC33277 and *Fusobacterium nucleatum* strain ATCC10953 were grown on blood agar anaerobically at 37°C as described previously (Gawron et al. 2014). Bacteria were next inoculated into brain–heart infusion (BHI) broth (Becton Dickinson) containing 0.5 mg/mL L-cysteine, 10 μg/mL hemin and 0.5 μg/mL vitamin K. Following anaerobic (85% $N_2$, 10% $CO_2$ and 5% $H_2$) overnight (o/n) culture, bacteria were resuspended in fresh BHI broth at optical density $(OD)_{600nm} = 0.1$ and cultured for an additional 20 h. After centrifugation and washing, bacteria were suspended in PBS to obtain a final concentration of $10^9$ colony-forming units (CFU)/mL and used for cell infection. GFs were treated with dimethyl sulfoxide (DMSO) (BioShop) or HDACi: SAHA (Abcam), ITF2357 (Italfarmaco), MS-275 (TargetMol) or a panel of selective HDACi synthesized at Italfarmaco: HDAC3/6i, HDAC6i(a), HDAC6i(b) or HDAC8(i). Details of HDACi specificity are shown in Appendix Table 2. After 30 min incubation with HDACi, cells were stimulated with TNFα (BioLegend) or infected with *P. gingivalis* or *F. nucleatum* at the indicated multiplicity of infection (MOI). In some experiments, protein synthesis was blocked with cycloheximide (TargetMol) before TNFα stimulation.

### *Statistical Analyses*

Data are presented as the mean ± SEM of independent experiments performed on GFs from different donors/patients unless otherwise indicated. Ratio paired *t* test or 1-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test were used for comparisons between groups. *P* values $<0.05$ were considered statistically significant.

Detailed descriptions of RNA isolation, quantitative polymerase chain reaction (PCR), Western blot analysis, enzyme-linked immunosorbent assay (ELISA), cell viability measurements, siRNA transfection, flow cytometry and colony-forming assays are provided in the online supplement and/or were described previously (Maksylewicz et al. 2019).

## Results

### *HDACi Differentially Regulate Protein Acetylation without Affecting Viability of GFs*

To identify the role of HDAC activity and involvement of individual HDAC family members in GF inflammatory and antimicrobial responses, we utilized a panel of pan-HDACi and inhibitors with distinct selectively profiles: MS-275 (class I-selective, but preferentially targeting HDAC1), specific

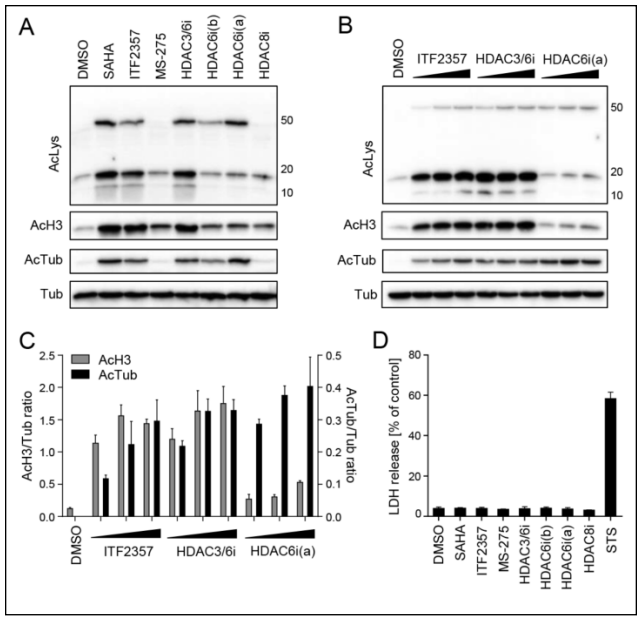


**Figure 1.** HDACi induce protein hyperacetylation without affecting viability of GFs from healthy donors. (**A, B**) Western blot analysis of total acetylated lysine (AcLys), acetyl-histone H3 (AcH3), acetyl-tubulin (AcTub) and total tubulin (Tub) in cell lysates of GFs (A) after 2 h treatment with DMSO or HDACi: SAHA (5 μM), ITF2357 (500 nM), MS-275 (2.5 μM), HDAC3/6i, HDAC6i(b), HDAC6i(a) or HDAC8i (all at 500 nM) or (B, **C**) after 2 h treatment with increasing concentrations (200 nM, 500 nM and 1 μM) of ITF2357, HDAC3/6i, HDAC6i(a). Results are shown as (A and B) representative of three independent experiments or (C) mean signal intensity from densitometric analysis (n = 3) +SEM. (**D**) Viability of GFs (n = 3) treated with DMSO or HDACi: SAHA (5 μM), ITF2357 (1 μM), MS-275 (2.5 μM), HDAC3/6i, HDAC6i(b), HDAC6i(a) or HDAC8i (all at 1 μM) for 24 h determined using LDH release assay and presented as % of control (cell lysate) +SEM. 1 μM staurosporine (STS) was used as a positive control. DMSO, dimethyl sulfoxide; HDAC, histone deacetylase; HDACi, HDAC inhibitor; GF, gingival fibroblast; SAHA, suberoylanilide hydroxamic acid.

inhibitors of HDAC6 (HDAC6i(a) and HDAC6i(b)) and HDAC8 (HDAC8i), and a compound that is a potent inhibitor HDAC3 and HDAC6 (designated as HDAC3/6i, though it also targets other HDACs at higher concentrations). GF treatment with selective and pan-HDACi resulted in distinct profiles of changes in global protein acetylation. The pan-HDACi SAHA and ITF2357 induced hyperacetylation of histone 3 (H3) and tubulin (Fig. 1A). A similar acetylation profile was observed in GFs treated with HDAC3/6i, whereas both HDAC6i selectively induced tubulin hyperacetylation. In contrast, MS-275 and HDAC8i had no effect on tubulin acetylation and only moderately increased the levels of acetylated H3 (Fig. 1A). Analysis of global protein acetylation using an acetyl-lysine antibody revealed a similar pattern of hyperacetylation upon HDACi treatment (Fig. 1A, top panel: 52kDa, 18kDa, and 11kDa bands correspond to tubulin, H3, and H4, respectively). Tubulin hyperacetylation was visible after treatment with 200 nM ITF2357, HDAC3/6i, and HDAC6i(a) and increased up to 500 nM or 1 μM HDACi concentration, whereas ITF2357- and HDAC3/6i-induced H3 acetylation was prominent at 200 nM and only marginally increased at higher concentrations (Fig. 1B, C). None of the tested HDACi affected GF viability after 24h treatment as determined by the lactate dehydrogenase (LDH) release assay (Fig. 1D) and annexin V binding (Appendix Fig. 1). These observations confirmed the pharmacological activity of the HDACi in GFs and excluded the possibility that compound cytotoxicity could influence our subsequent analyses.

## *Pan-HDACi and HDAC3/6i Suppress TNFα-Induced Inflammatory Gene Expression in GFs*

To assess the involvement of HDACs in GF inflammatory activation, we stimulated GFs from healthy individuals with TNFα in the absence or presence of the previously developed panel of HDACi and analyzed expression of a cluster of mediators that are sensitive to manipulation of protein acetylation (Maksylewicz et al. 2019). SAHA (5 μM) and ITF2357 (500 nM) reduced TNFα-induced mRNA expression of *CCL2, CCL5, CXCL10*, and *IL1B* by >80%. Both pan-HDACi also suppressed *COX2* expression by 60%, but had no effect on *IL8* induction by TNFα (Fig. 2A). GF treatment with HDAC3/6i, but not with HDAC6i(a) or HDAC6i(b) (all at 500 nM), resulted in similar effects on inflammatory gene induction by TNFα as those observed with pan-HDACi. MS-275 moderately reduced *CXCL10* expression induced by TNFα, while leaving other genes unaffected. In contrast, HDAC8i had no effect on any of the analyzed transcripts (Fig. 2A). To test whether HDACi effects on mRNA expression correspond to changes at the protein level, we treated GFs with increasing concentrations of ITF2357, HDAC3/6i, and HDAC6i(a) before stimulation with TNFα for 24 h. ITF2357 and HDAC3/6i dose-dependently reduced TNFα-induced CCL2 production, reaching >50% reduction of the CCL2 protein level at 1 μM ($P < 0.001$). HDAC6i(a) failed to affect CCL2 secretion regardless of the compound concentration (Fig. 2B). Consistent with mRNA data, ITF2357 and HDAC3/6i, but not HDAC6i(a) (all at 500 nM), also significantly suppressed CCL5 and CXCL10, while IL-8 secretion remained unaffected (Fig. 2C). Together, these experiments identified a key role of HDAC activity in transcriptional induction of a cluster of inflammatory mediators in GFs and suggest that HDAC3/6i effects on GF activation can be predominantly attributed to its effects on HDAC3.

## *Anti-Inflammatory Effects of HDACi Are Not Caused by Induction of Corepressors or Modulation of Signaling Pathways*

To verify whether the anti-inflammatory properties of HDACi are mediated by induction of transcriptional corepressors, we treated GFs with SAHA in combination with the protein translation inhibitor cycloheximide (CHX) prior to TNFα stimulation. However, both in the absence or presence of CHX, SAHA reduced transcriptional induction of *CCL2, IL1B*, and *COX2* to a similar degree (Fig. 3A), suggesting that gene suppression by HDACi does not require de novo synthesis of putative repressors. Subsequently, we tested if HDACi affect signaling pathways critical for GF inflammatory activation: mitogen-activated protein kinase (MAPK) and NF-κB signaling, both of which

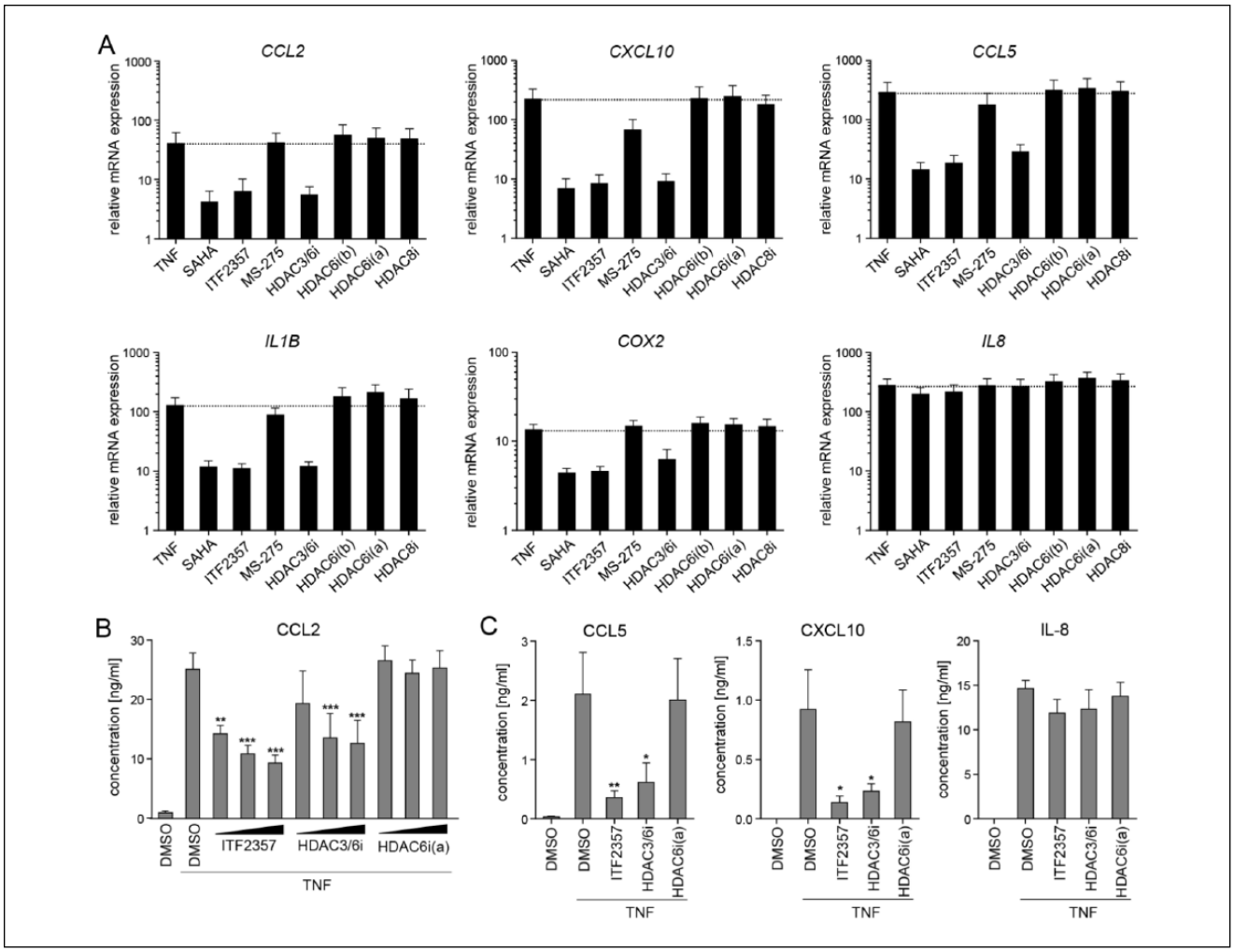


**Figure 2.** HDACi suppress TNFα-induced expression of inflammatory mediators in GFs from healthy donors. (**A**) qPCR analysis of *CCL2, CXCL10, CCL5, IL1B, COX2* and *IL8* expression in GFs ($n$ = 5) stimulated with 10 ng/ml TNFα for 4 h after 30 min treatment with DMSO or HDACi: SAHA (5 μM), ITF2357 (500 nM), MS-275 (2.5 μM), HDAC3/6i, HDAC6i(b), HDAC6i(a), or HDAC8i (all at 500 nM). Results are shown as mean relative expression + SEM. (**B**) CCL2 secretion by GFs ($n$ = 5) treated with DMSO or increasing concentrations (200 nM, 500 nM, and 1 μM) of ITF2357, HDAC3/6i, and HDAC6i(a) for 30 min prior to stimulation with 10 ng/ml TNFα for 24 h. (**C**) CCL5, CXC10, and IL-8 secretion by GFs ($n$ = 4–5) treated with DMSO or HDACi: ITF2357, HDAC3/6i, and HDAC6i(a) (all at 500 nM) for 30 min before stimulation with 10 ng/ml TNFα for 24 h. (B) and (C) Data are presented as mean concentration + SEM. Dotted line in (A) indicates expression levels in control TNFα-stimulated samples. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; 1-way ANOVA followed by Bonferroni multiple comparison test. DMSO, dimethyl sulfoxide; HDAC, histone deacetylase; HDACi, HDAC inhibitor; GF, gingival fibroblast; qPCR, quantitative polymerase chain reaction; SAHA, suberoylanilide hydroxamic acid.

are acetylation-dependent. Neither ITF2357 nor HDAC3/6i, had any significant effect on TNFα-induced p38 and extracellular signal-regulated kinase (ERK) MAPK phosphorylation, IκBα proteasomal degradation, and Ser536 phosphorylation of the p65 NFκB subunit (Fig. 3B), demonstrating that the observed anti-inflammatory activity of HDACi is not related to their direct effects on early signaling events in GFs.

## *HDAC3 Is a Key Regulator of Inflammatory Gene Expression in* P. gingivalis*–Infected GFs*

In the inflamed gingival tissue, oral pathogens penetrate the epithelial layer and invade the connective tissue, directly interacting with resident cells. We thus investigated HDAC contributions to inflammatory and antimicrobial responses of GFs from healthy donors to *P. gingivalis* infection. Treatment with the pan-HDACi ITF2357 or HDAC3/6i prior to *P. gingivalis* infection (MOI = 100) significantly reduced the induction of several inflammatory mediators that contribute to the pathogenesis of periodontitis (Yucel-Lindberg and Båge 2013) and are regulated by *P. gingivalis* infection, including *CCL2, CCL5, CXCL10, IL1B, COX2*, and *MMP3* mRNA (Fig. 4A). Trends towards reduced expression of *MMP1* and *IL8* were also noted, while *IL6* mRNA levels were unaffected by HDACi. In contrast, HDAC6i(a) had no effect on any of the analyzed genes (Fig. 4A). Similar effects of ITF2357 and/or HDAC3/6i on gene expression were observed in GFs infected with *P. gingivalis* at lower MOIs or with another member of the bacterial

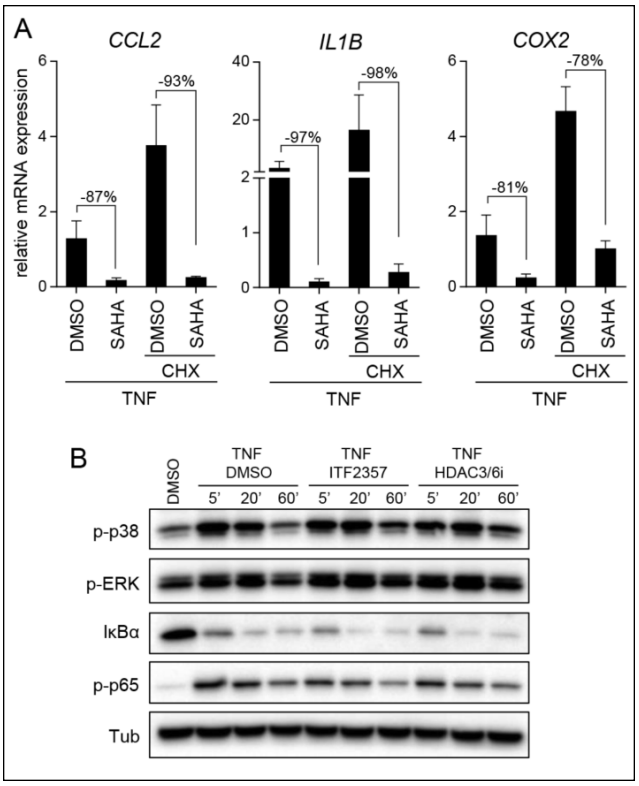


**Figure 3.** MAPK and NFκB signaling is unaffected by HDACi and *de novo* protein synthesis is not required for anti-inflammatory effects of HDACi in GFs from healthy individuals. (**A**) qPCR analysis of relative mRNA levels of *CCL2, IL1B* and *COX2* in GFs ($n = 4$) incubated with DMSO or 5 μM SAHA in the presence or absence of cycloheximide (CHX) for 30 min before stimulation with TNFα (10 ng/ml) for 4h. Data are shown as mean +SEM; % of suppression relative to DMSO control are depicted in each graph. (**B**) Western blot analysis of phospho (p)-p38, p-ERK, IκBα, p-p65(Ser536) and tubulin (Tub) in GF total cell lysates after treatment with DMSO, ITF2357 or HDAC3/6i (both at 500 nM) for 30 min prior to stimulation with TNFα (10 ng/ml) for 5, 20 or 60 min. Results representative of 3 independent experiments are shown. DMSO, dimethyl sulfoxide; HDAC, histone deacetylase; HDACi, HDAC inhibitor; GF, gingival fibroblast; MAPK, mitogen-activated protein kinase; qPCR, quantitative polymerase chain reaction; SAHA, suberoylanilide hydroxamic acid.

periodontal community, *F. nucleatum* (Appendix Fig. 2). Consistent with mRNA data, *P. gingivalis*-induced CCL2 protein secretion was dose-dependently suppressed by HDAC3/6i, but not by HDAC6i(a) (Fig. 4B). To exclude the possibility that the observed effects could be attributed to other HDACs targeted by HDAC3/6i, we silenced HDAC3 expression in GFs using specific siRNA. Knockdown efficiency was confirmed at the mRNA and protein level (Fig. 4C, D). HDAC3 silencing significantly suppressed *P. gingivalis*–mediated upregulation of the genes targeted by HDAC3/6i, including *CCL2* and *IL1B* (Fig. 4E). A trend toward reduced *MMP3* and *COX2* induction was also noted, though it did not reach statistical significance (Fig. 4E). Notably, HDAC1 knockdown had negligible effect on *P. gingivalis*–induced inflammatory gene expression (Appendix Fig. 3), confirming the specific role of HDAC3 in this process.

We also tested if HDAC inhibition affects *P. gingivalis* adhesion, invasion, and intracellular survival. Treatment of GFs with SAHA or ITF2357 failed to affect the load of the CFSE-labelled bacteria detected by flow cytometry, or the numbers of bacterial colonies determined using the colony-forming assay after 1 h of infection (Fig. 4F–H). Pan-HDACi also had no effect on the numbers of live bacteria detected in GF lysates 24 h post infection (Fig. 4H) or after 1 h of treatment with antibiotics to eliminate adherent bacteria that were not internalized (Fig. 4I). Together, these results suggest that HDAC activity is not required for elimination of intracellular *P. gingivalis*, but identify HDAC3 as a prominent regulator of GF inflammatory activation.

### *GFs from Periodontitis Patients are Sensitive to the Anti-inflammatory Effects of HDACi*

GFs isolated from the site of gingival inflammation maintain an activated phenotype in culture and are hyperresponsive to infection with *P. gingivalis* (Scheres et al. 2011; Baek et al. 2013). To verify whether GFs extracted from the inflamed gingival tissue were sensitive to the anti-inflammatory effects of HDACi, we treated GFs from 4 periodontitis patients with ITF2357, HDAC3/6i, or HDAC6i(a) prior to *P. gingivalis* infection. ITF2357 and HDAC3/6i, but not HDAC6i(a), suppressed the induction of *CCL2, CCL5, CXCL10, IL1B, MMP3*, and *MMP1* to a similar degree as in GFs from healthy individuals (Fig. 5). The effect of HDACi on *P. gingivalis*–induced *COX2* expression was variable and patient-dependent, whereas *IL8* and *IL6* mRNA levels were largely unaffected by any of the tested HDACi, in line with data from healthy donor GFs (Fig. 5). Together, these findings demonstrate that HDAC activity is essential for transcriptional induction of an important cluster of inflammatory mediators in GFs and suggest that selective targeting of HDAC3 could dampen the chronic inflammatory mediator production in periodontitis.

## Discussion

The balance between protein acetylation and deacetylation is dysregulated in many chronic inflammatory diseases and targeting these alterations with HDACi or acetylated histone mimetics that inhibit BET bromodomain proteins has strong immunomodulatory effects (Tough et al. 2016). In experimental periodontitis, HDACi and BET inhibitors were found to exert anti-inflammatory and/or bone-protective activity (Cantley et al. 2011; Meng et al. 2014), highlighting the importance of protein acetylation in inflammation and bone homeostasis. Here, we show for the first time that HDAC activity is essential for transcriptional induction of a plethora of inflammatory genes, including chemokines and MMPs, in GFs derived from healthy and periodontitis-affected individuals. Many of these mediators are present in large quantities in gingival crevicular fluid and/or the gingival tissue from patients with periodontal disease, significantly contributing to pathology (Yucel-Lindberg and Båge 2013). Consequently, they represent important targets for development of new host modulation therapies

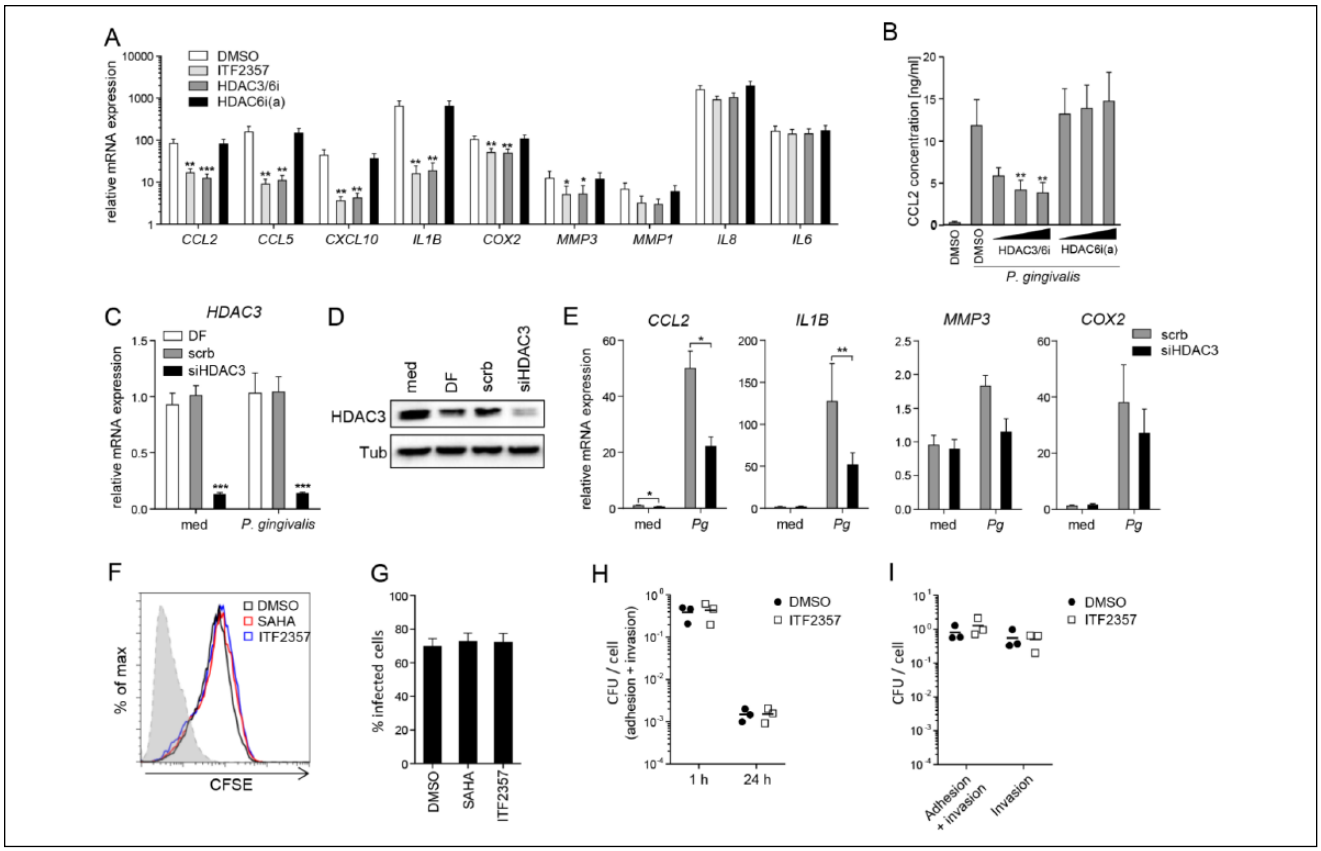


**Figure 4.** HDAC3 inhibition or silencing suppresses inflammatory gene expression in GFs from healthy donors infected with *P. gingivalis*. (**A**) qPCR analysis of *CCL2, CCL5, CXCL10, IL1B, COX2, MMP3, MMP1, IL8*, and *IL6* mRNA levels in GFs ($n = 6$) treated with DMSO or HDACi: ITF2357, HDAC3/6i, HDAC6i(a) (all at 500 nM) for 30 min prior to 4 h infection with *P. gingivalis* (MOI = 100). (**B**) CCL2 production by GFs ($n = 4$) exposed to DMSO or treated with HDAC3/6i or HDAC6i(a) at 200 nM, 500 nM, or 1 µM for 30 min before *P. gingivalis* infection (MOI = 100) for 1 h, followed by washing and 23 h culture in fresh medium containing DMSO or HDACi (mean concentration + SEM). (**C**) HDAC3 mRNA ($n = 4$) and (**D**) protein expression in GFs transfected with transfection reagent alone (Dharmafect, DF), control non targeting siRNA (scrb) or HDAC3-specific siRNA (siHDAC3) in the absence (medium, med) or presence of infection with *P. gingivalis* (MOI = 100) for 4 h. Western blots for HDAC3 and tubulin (Tub) representative of 2 independent experiments are shown. (**E**) Relative mRNA expression of *CCL2, IL1B, MMP3*, and *COX2* in GFs ($n = 4$-5) transfected with non targeting siRNA (scrb) or siHDAC3 prior to infection with *P. gingivalis* (*Pg*) (MOI = 100) for 4 h. qPCR data in (A), (C), and (E) are shown as the mean relative expression + SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; (A) and (C) one-way ANOVA followed by Bonferroni multiple comparison test; (E) ratio paired t test. (**F, G**) Adhesion and internalization of *P. gingivalis* by GFs treated with SAHA (5 µM) or ITF2357 (500 nM) for 30 min before infection with CFSE-labelled *P. gingivalis* (MOI = 100) determined by flow cytometry. (F) A representative histogram and (G) mean % of infected cells of two independent experiments are shown. (**H**) Adhesion and invasion of *P. gingivalis* in GFs after treatment with DMSO or 500 nM ITF2357 prior to infection with *P. gingivalis* (MOI = 100) for 1 h determined by colony-forming assay immediately (1 h) or 24 h post infection. (**I**) Adhesion and invasion of *P. gingivalis* in GFs treated as in (H) followed by 1 h of infection with *P. gingivalis* and subsequent incubation for 1 h with or without antibiotics. (H, I) Results are shown as mean colony-forming units (CFU)/cell of 3 independent experiments. DMSO, dimethyl sulfoxide; HDAC, histone deacetylase; HDACi, HDAC inhibitor; GF, gingival fibroblast; MAPK, mitogen-activated protein kinase; qPCR, quantitative polymerase chain reaction; SAHA, suberoylanilide hydroxamic acid.

aimed at amelioration of the destructive aspects of the host immune response to oral pathogens (Preshaw 2018). To this end, our data indicate that HDACi may be useful in suppressing the excessive production of these mediators.

Individual HDAC family members play unique and non-redundant roles in distinct immunological processes. In macrophages, specific inhibition of HDAC6 promotes killing of intracellular *Salmonella enterica* serovar Typhimurium (Ariffin et al. 2015), HDAC8 targeting restores immune responses during *Bacillus anthracis* infection (Ha et al. 2016) and HDAC1 silencing prevents intracellular propagation of *Anaplasma phagocytophilum* (Garcia-Garcia et al. 2009). However, important contributions of these HDACs to inflammatory responses seem to be largely restricted to immune cells. In our experiments, we found no evidence to support involvement of HDAC1, HDAC6, or HDAC8 in GF activation, consistent with observations in synovial fibroblasts showing that selective targeting of these HDACs had little effect on cytokine expression (Angiolilli et al. 2017).

HDAC3 has been previously identified as an important epigenetic regulator of inflammatory gene transcription. Genetic depletion of HDAC3 in macrophages suppresses LPS-inducible gene expression and promotes an anti-inflammatory wound healing phenotype that is beneficial in the context of

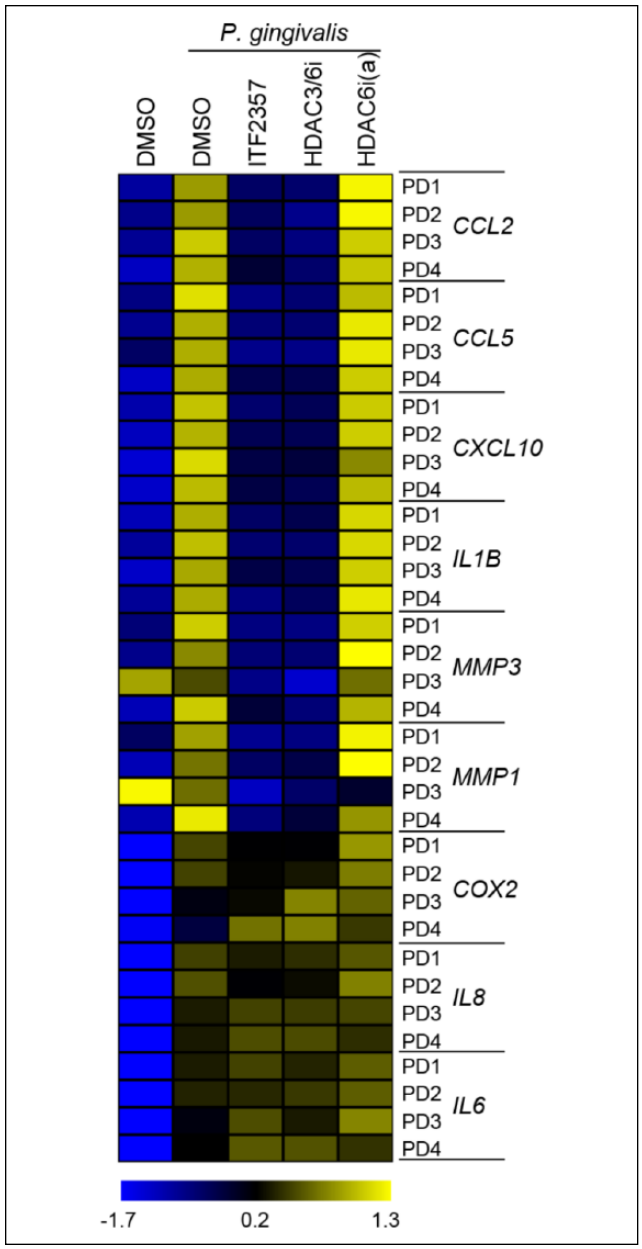


**Figure 5.** GFs from patients with periodontitis are sensitive to the anti-inflammatory activity of global and HDAC3-selective HDACi. GFs from periodontitis patients ($n$ = 4) were treated with DMSO, ITF2357, HDAC3/6i, or HDAC6i(a) (all at 500 nM) for 30 min prior to infection with *P. gingivalis* (MOI = 100) for 4 h and mRNA expression of inflammatory mediators was analyzed by qPCR. Data for individual patients (PD1–PD4) are shown on a heat map as row Z-scores calculated from ΔCt values relative to a housekeeping gene (RPLP0). DMSO, dimethyl sulfoxide; HDAC, histone deacetylase; HDACi, HDAC inhibitor; GF, gingival fibroblast; qPCR, quantitative polymerase chain reaction.

atherosclerosis (Chen et al. 2012; Hoeksema et al. 2014). Selective HDAC3 inhibition has been shown to reduce cytokine production by peripheral blood mononuclear cells and synovial fibroblasts from RA patients (Gillespie et al. 2012; Angiolilli et al. 2017). Our findings that HDAC3 knockdown and HDAC3/6i, but not HDAC6i, suppressed cytokine- and *P. gingivalis*–induced expression of a subset of inflammatory genes in GFs support a model in which HDAC3 is a primary regulator of transcription during inflammatory response. Although the cluster of analyzed genes was too small to draw any conclusions about specific gene networks regulated by HDAC3 in GFs, other reports suggest that the anti-inflammatory effects of HDAC3 targeting are partly dependent on the suppression of the type I interferon autocrine/paracrine activity (Chen et al. 2012; Angiolilli et al. 2017). The involvement of interferon signaling could explain differential regulation of *CXCL10* and *IL8* expression by HDACi, which is surprising given that these genes are located on the same chromosome in close proximity. Future studies should verify whether this mechanism is operational in GFs, particularly in light of recent evidence implicating excessive type I interferon production as the cause of alveolar bone loss in periodontitis (Mizraji et al. 2017).

HDACi mediate their anti-inflammatory effects through epigenetic and non epigenetic mechanisms. Proteomic studies identified acetylation sites on more than 1,700 proteins (Choudhary et al. 2009) and reversible acetylation of transcription factors and components of the signal transduction machinery plays a critical role during inflammation (Grabiec et al. 2011). Acetylation-mediated MAPK phosphatase-1 activation suppresses p38 signaling (Cao et al. 2008), whereas NFκB function is regulated by acetylation of several distinct lysine residues on p65 and p50 subunits, affecting their nuclear retention, DNA binding affinity, and transcriptional activity (Ghizzoni et al. 2011). However, consistent with previous observations in synovial fibroblasts (Grabiec et al. 2012), we failed to identify any effect of HDACi on p38 and ERK phosphorylation, IκBα degradation, or NFκB p65 activation, arguing against direct regulation of these pathways in GFs by HDACs. Our observation that blocking of de novo protein synthesis had no effect on HDACi-mediated gene suppression also demonstrates that the identified effects could not be attributed to transcriptional induction of repressor proteins, which is a known indirect HDACi mechanism of action (Tang and Goldman 2006). Additional studies are needed to fully understand the molecular events underlying the anti-inflammatory effects of HDACi in gingival cells, including analyses of local changes in the histone acetylation status at the promoters of HDACi-sensitive genes and posttranscriptional regulatory mechanisms. The latter will be of particular interest in light of studies demonstrating that changes in mRNA stability are partly responsible for the anti-inflammatory effects of HDACi in synovial fibroblasts (Grabiec et al. 2012; Angiolilli et al. 2018). Notably, mRNA destabilization by HDACi is mediated by the RNA-binding protein tristetraprolin (Angiolilli et al. 2018), which is an important regulator of alveolar bone homeostasis (Steinkamp et al. 2018). The potential HDACi off-target effects also need to be considered. Although chemoproteomic profiling demonstrated selective targeting of HDACi to HDAC-containing protein complexes, these compounds may also affect other zinc-dependent metalloenzymes with a similar chemical ligand space (Bantscheff et al. 2011).

While HDACi effects on GF activation require further characterization with regards to the involvement of epigenetic and non epigenetic mechanisms, and should be studied in detail also in gingival epithelial cells (Yin and Chung 2011), the results presented here provide strong evidence that HDAC3 acts as a crucial regulator of inflammatory mediator expression. Our data suggest that HDACi, in particular selective HDAC3 inhibitors (Bresciani et al. 2019), may ameliorate the excessive inflammatory response of gingival cells to periodontal pathogens, and their application could be clinically beneficial as an adjunct to the conventional treatment of periodontitis.

## Author Contributions

K.B. Lagosz, A. Bysiek, J.M. Macina, contributed to design, data acquisition, analysis, and interpretation, critically revised the manuscript; G.P. Bereta, M. Kantorowicz, W. Lipska, contributed to data acquisition, critically revised the manuscript; M. Sochalska, K. Gawron, contributed to data analysis and interpretation, critically revised the manuscript; T. Kaczmarzyk, M. Chomyszyn-Gajewska, G. Fossati, J. Potempa, contributed to conception and design, critically revised the manuscript; A.M. Grabiec, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

## Acknowledgments

We thank Dr. Pawel A. Kabala (University Medical Center Utrecht, The Netherlands) for help with heat map preparation and Ms. Marta Kaminska (Jagiellonian University) for assistance in flow cytometry. This work was supported by a research grant from the National Science Centre, Poland to A.M.G. (POLONEZ fellowship 2015/19/P/NZ7/03659; this project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 665778). A.M.G. and M.S. are also supported by the Foundation for Polish Science program FIRST TEAM co-financed by the European Union under the European Regional Development Fund (grant numbers POIR.04.04.00-00-5EDE/18-00 to A.M.G. and First Team-4/2017/40 to M.S.). Other funding included grants from the National Science Centre, Poland (UMO-2018/30/A/NZ5/00650 to J.P. and UMO-2018/29/B/NZ2/01930 to K.G.) and from The National Institutes of Health/National Institute of Dental and Craniofacial Research, USA to J.P. (R01 DE 022597). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

## ORCID iDs

G.P. Bereta https://orcid.org/0000-0002-2925-7796
K. Gawron https://orcid.org/0000-0003-3430-6270
A.M. Grabiec https://orcid.org/0000-0001-9472-850X

## References

Angiolilli C, Kabala PA, Grabiec AM, Rossato M, Lai WS, Fossati G, Mascagni P, Steinkühler C, Blackshear PJ, Reedquist KA, et al. 2018. Control of cytokine mRNA degradation by the histone deacetylase inhibitor ITF2357 in rheumatoid arthritis fibroblast-like synoviocytes: beyond transcriptional regulation. Arthritis Res Ther. 20(1):148.

Angiolilli C, Kabala PA, Grabiec AM, Van Baarsen IM, Ferguson BS, García S, Malvar Fernandez B, McKinsey TA, Tak PP, Fossati G, et al. 2017. Histone deacetylase 3 regulates the inflammatory gene expression programme of rheumatoid arthritis fibroblast-like synoviocytes. Ann Rheum Dis. 76(1):277–285.

Ariffin JK, das Gupta K, Kapetanovic R, Iyer A, Reid RC, Fairlie DP, Sweet MJ. 2015. Histone deacetylase inhibitors promote mitochondrial reactive oxygen species production and bacterial clearance by human macrophages. Antimicrob Agents Chemother. 60(3):1521–1529.

Baek KJ, Choi Y, Ji S. 2013. Gingival fibroblasts from periodontitis patients exhibit inflammatory characteristics in vitro. Arch Oral Biol. 58(10):1282–1292.

Bannister AJ, Kouzarides T. 2011. Regulation of chromatin by histone modifications. Cell Res. 21(3):381–395.

Bantscheff M, Hopf C, Savitski MM, Dittmann A, Grandi P, Michon AM, Schlegl J, Abraham Y, Becher I, Bergamini G, et al. 2011. Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes. Nat Biotechnol. 29(3):255–265.

Benakanakere M, Kinane DF. 2012. Innate cellular responses to the periodontal biofilm. Front Oral Biol. 15:41–55.

Bresciani A, Ontoria JM, Biancofiore I, Cellucci A, Ciammaichella A, Di Marco A, Ferrigno F, Francone A, Malancona S, Monteagudo E, et al. 2019. Improved selective class I HDAC and novel selective HDAC3 inhibitors: beyond hydroxamic acids and benzamides. ACS Med Chem Lett. 10(4):481–486.

Cantley MD, Bartold PM, Marino V, Fairlie DP, Le GT, Lucke AJ, Haynes DR. 2011. Histone deacetylase inhibitors and periodontal bone loss. J Periodontal Res. 46(6):697–703.

Cantley MD, Dharmapatni AA, Algate K, Crotti TN, Bartold PM, Haynes DR. 2016. Class I and II histone deacetylase expression in human chronic periodontitis gingival tissue. J Periodontal Res. 51(2):143–151.

Cao W, Bao C, Padalko E, Lowenstein CJ. 2008. Acetylation of mitogen-activated protein kinase phosphatase-1 inhibits Toll-like receptor signaling. J Exp Med. 205(6):1491–503.

Chen X, Barozzi I, Termanini A, Prosperini E, Recchiuti A, Dalli J, Mietton F, Matteoli G, Hiebert S, Natoli G. 2012. Requirement for the histone deacetylase Hdac3 for the inflammatory gene expression program in macrophages. Proc Natl Acad Sci USA. 109(42):E2865–E2874.

Choi SW, Braun T, Chang L, Ferrara JLM, Pawarode A, Magenau JM, Hou G, Beumer JH, Levine JE, Goldstein S, et al. 2014. Vorinostat plus tacrolimus and mycophenolate to prevent graft-versus-host disease after related-donor reduced-intensity conditioning allogeneic haemopoietic stem-cell transplantation: a phase 1/2 trial. Lancet Oncol. 15(1):87–95.

Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen J V, Mann M. 2009. Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science. 325(5942):834–840.

Garcia-Garcia JC, Barat NC, Trembley SJ, Dumler JS. 2009. Epigenetic silencing of host cell defense genes enhances intracellular survival of the rickettsial pathogen Anaplasma phagocytophilum. PLoS Pathog. 5(6):e1000488.

Gawron K, Bereta G, Nowakowska Z, Lazarz-Bartyzel K, Lazarz M, Szmigielski B, Mizgalska D, Buda A, Koziel J, Oruba Z, et al. 2014. Peptidylarginine deiminase from Porphyromonas gingivalis contributes to infection of gingival fibroblasts and induction of prostaglandin E2 -signaling pathway. Mol Oral Microbiol. 29(6):321–332.

Ghizzoni M, Haisma HJ, Maarsingh H, Dekker FJ. 2011. Histone acetyltransferases are crucial regulators in NF-κB mediated inflammation. Drug Discov Today. 16(11–12):504–511.

Gillespie J, Savic S, Wong C, Hempshall A, Inman M, Emery P, Grigg R, McDermott MF. 2012. Histone deacetylases are dysregulated in rheumatoid arthritis and a novel histone deacetylase 3-selective inhibitor reduces interleukin-6 production by peripheral blood mononuclear cells from rheumatoid arthritis patients. Arthritis Rheum. 64(2):418–422.

Grabiec AM, Korchynskyi O, Tak PP, Reedquist KA. 2012. Histone deacetylase inhibitors suppress rheumatoid arthritis fibroblast-like synoviocyte and macrophage IL-6 production by accelerating mRNA decay. Ann Rheum Dis. 71(3):424–431.

Grabiec AM, Potempa J. 2018. Epigenetic regulation in bacterial infections: targeting histone deacetylases. Crit Rev Microbiol. 44(3):336–350.

Grabiec AM, Tak PP, Reedquist KA. 2011. Function of histone deacetylase inhibitors in inflammation. Crit Rev Immunol. 31(3):233–263.

Ha SD, Reid C, Meshkibaf S, Kim SO. 2016. Inhibition of interleukin 1β (IL-1β) expression by anthrax lethal toxin (LeTx) is reversed by histone deacetylase 8 (HDAC8) inhibition in murine macrophages. J Biol Chem. 291(16):8745–8755. Erratum in: J Biol Chem. 2016 291(44): 23363.

Hajishengallis G. 2014. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. Trends Immunol. 35(1):3–11.

Hoeksema MA, Gijbels MJ, Van den Bossche J, van der Velden S, Sijm A, Neele AE, Seijkens T, Stöger JL, Meiler S, Boshuizen MC, et al. 2014. Targeting macrophage Histone deacetylase 3 stabilizes atherosclerotic lesions. EMBO Mol Med. 6(9):1124–1132.

Leoni F, Fossati G, Lewis EC, Lee JK, Porro G, Pagani P, Modena D, Moras ML, Pozzi P, Reznikov LL, et al. 2005. The histone deacetylase inhibitor ITF2357 reduces production of pro-inflammatory cytokines in vitro and systemic inflammation in vivo. Mol Med. 11(1–12):1–15.

Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P, Donà G, Fossati G, Sozzani S, Azam T, et al. 2002. The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. Proc Natl Acad Sci USA. 99(5):2995–3000.

Maksylewicz A, Bysiek A, Lagosz KB, Macina JM, Kantorowicz M, Bereta G, Sochalska M, Gawron K, Chomyszyn-Gajewska M, Potempa J, et al. 2019. BET bromodomain inhibitors suppress inflammatory activation of gingival fibroblasts and epithelial cells from periodontitis patients. Front Immunol. 10:933.

Martins MD, Jiao Y, Larsson L, Almeida LO, Garaicoa-Pazmino C, Le JM, Squarize CH, Inohara N, Giannobile WV, Castilho RM. 2016. Epigenetic modifications of histones in periodontal disease. J Dent Res. 95(2):215–222.

Meng S, Zhang L, Tang Y, Tu Q, Zheng L, Yu L, Murray D, Cheng J, Kim SH, Zhou X, et al. 2014. BET inhibitor JQ1 blocks inflammation and bone destruction. J Dent Res. 93(7):657–662.

Mizraji G, Nassar M, Segev H, Sharawi H, Eli-Berchoer L, Capucha T, Nir T, Tabib Y, Maimon A, Dishon S, et al. 2017. Porphyromonas gingivalis promotes unrestrained type I interferon production by dysregulating TAM signaling via MYD88 degradation. Cell Rep. 18(2):419–431.

Preshaw PM. 2018. Host modulation therapy with anti-inflammatory agents. Periodontol 2000. 76(1):131–149.

Scheres N, Laine ML, Sipos PM, Bosch-Tijhof CJ, Crielaard W, de Vries TJ, Everts V. 2011. Periodontal ligament and gingival fibroblasts from periodontitis patients are more active in interaction with Porphyromonas gingivalis. J Periodontal Res. 46(4):407–416.

Steinkamp HM, Hathaway-Schrader JD, Chavez MB, Aartun JD, Zhang L, Jensen T, Shojaee Bakhtiari A, Helke KL, Stumpo DJ, Alekseyenko AV, et al. 2018. Tristetraprolin is required for alveolar bone homeostasis. J Dent Res. 97(8):946–953.

Tang H, Goldman D. 2006. Activity-dependent gene regulation in skeletal muscle is mediated by a histone deacetylase (HDAC)-Dach2-myogenin signal transduction cascade. Proc Natl Acad Sci USA. 103(45):16977–16982.

Tough DF, Tak PP, Tarakhovsky A, Prinjha RK. 2016. Epigenetic drug discovery: breaking through the immune barrier. Nat Rev Drug Discov. 15(12):835–853.

Vojinovic J, Damjanov N, D'Urzo C, Furlan A, Susic G, Pasic S, Iagaru N, Stefan M, Dinarello CA. 2011. Safety and efficacy of an oral histone deacetylase inhibitor in systemic-onset juvenile idiopathic arthritis. Arthritis Rheum. 63(5):1452–1458.

Yang XJ, Seto E. 2008. The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. Nat Rev Mol Cell Biol. 9(3):206–218.

Yin L, Chung WO. 2011. Epigenetic regulation of human β-defensin 2 and CC chemokine ligand 20 expression in gingival epithelial cells in response to oral bacteria. Mucosal Immunol. 4(4):409–419.

Yucel-Lindberg T, Båge T. 2013. Inflammatory mediators in the pathogenesis of periodontitis. Expert Rev Mol Med. 15:e7.

Zhang Z, Zhang R. 2015. Epigenetics in autoimmune diseases: pathogenesis and prospects for therapy. Autoimmun Rev. 14(10):854–863.